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ARTICLE

In Vivo Dose Threshold Effect of Adenovirus-Mediated Factor VIII Gene Therapy in Hemophiliac Mice

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While much is known about adenovirus biology from its development as a therapeutic gene delivery vehicle, an important question remains regarding the appropriate in vivo vector dose. We describe here an *in vivo* dose threshold effect with an adenoviral vector expressing human Factor VIII (FVIII) in hemophiliac mice. Upon administration of vector doses between 6×10^{10} and 2×10^{10} vector particles per mouse, FVIII was expressed linearly, whereas a dose of 1×10^{10} vector particles per mouse did not result in detectable levels of FVIII activity. In contrast, *in vitro* transduction studies demonstrated linear transgene expression over 2 to 3 log units. To further define this dose threshold effect, a vector-mixing study was performed. Mice were injected with a total vector dose of 6×10^{10} particles containing admixtures of FVIII vector plus a control vector lacking a transgene (null vector). With the admixture, FVIII activity was detected in mice that received 1×10^{10} particles of the FVIII vector, indicating that maintenance of the total viral input at 6×10^{10} particles per mouse circumvented the threshold dose effect. This threshold dose effect could not be attributed to dose-dependent differences in liver toxicity nor to dose-dependent induction of cellular and humoral immune responses. Southern blot analysis of livers revealed that mice receiving the vector admixture contained FVIII DNA, accounting for the observed FVIII expression, whereas mice receiving 1×10^{10} particles of FVIII vector had barely detectable FVIII DNA. These results suggest that the threshold effect is an *in vivo* phenomenon that will have important implications in defining the therapeutic window of adenoviral vectors for clinical applications.

Key Words: **nonreplicating; adenovirus; gene therapy; Factor VIII; hemophilia; threshold; *in vivo*; murine; dose; immunology.**

INTRODUCTION

Human adenoviruses are widely employed as gene therapy vehicles. Through the study and development of adenovirus as a recombinant vector *in vitro,* much is known about adenovirus biology, and analysis of dozens of serotypes has revealed valuable information about its genetic organization, virion structure, and life cycle (1–3). As with all viruses currently evaluated as gene delivery vehicles, the use of adenovirus for *in vivo* gene therapy has demonstrated a lack of sufficient understanding of adenoviral vectors with respect to optimal dose, route of delivery, tissue targeting, efficacy of cellular transduction, stability of transgene expression, and control of immune responses to adenovirus antigens and the transgene product (4–6).

The challenge to improve adenoviruses for gene therapy applications has been met by the development of vectors with deleted genetic regions (E1, E2, E3, E4, or gutless) to both accommodate transgenes and to reduce toxicity and immunogenicity (7–15). To propagate these attenuated adenoviral vectors, packaging cell lines have been created which replace the essential gene products *in trans* or, helper viruses are added to the cultures (7, 16). Adenoviruses have also been engineered to contain tissue-specific promoters to drive transgene expression (17–20) and to express altered capsid proteins to allow cell type-specific targeting and escape from neutralizing antibodies (21, 22). Through this research and development, some further attenuated adenoviral vectors were shown to have reduced toxicity in animals (10, 12, 15,

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23), and have resulted in long term transgene expression in some disease models such as hemophilia (17, 37).

Adenovirus administration in animal models has been evaluated for the induction of immunity to the vector or its transgene (24–32). In general, exposure to adenovirus results in the activation of a potent humoral response to adenovirus capsid proteins (30–32), although the dominant antibody epitopes have yet to be defined. In some experimental animal systems, adenovirus also induces a cellular immune response to immunogenic backbone gene products (24–26), capable of eliminating cells that have been transduced by adenovirus and present adenovirus-specific antigens. Although preexisting or induced immune responses can greatly diminish the practicality and efficacy of using adenoviruses to treat some diseases *in vivo,* there are promising new strategies being tested which aim to prevent or downregulate adenovirus-directed immunity.

In this report, we describe an *in vivo* threshold dose effect in hemophilia A mice using an E1/E2a/E3-deleted human adenovirus encoding human coagulation Factor VIII (FVIII), an effect that was not similarly observed *in vitro*. The threshold effect is defined as small incremental changes of input vector dose which result in disproportionate responses, in contrast to small incremental changes of input vector dose which result in proportional responses. This *in vivo* effect cannot be explained by evidence of dose-dependent hepatotoxicity, nor cellular or humoral immune responses since all doses of adenovirus used in this study induced similar toxicity profiles or immune responses. However, Southern blot analysis suggested that higher *in vivo* doses of adenoviral vectors may be necessary for efficient liver cell transduction to occur. These findings have important relevance to the application of adenoviruses in clinical gene therapy applications.

MATERIALS AND METHODS

Hemophiliac mouse procedures. Hemophilia A mice (a hybrid population of C57Bl/6 and S129 mouse strains) were generated by disruption of exon 16 or exon 17 of the murine FVIII gene (33). These mice were maintained as an inbred colony at our animal facility and were approximately 6–8 weeks old when experiments began. Adenovirus vectors were diluted in HBSS before injection into mice intravenously (iv) via tail vein, followed by application of topical thrombin (Thrombostat, Parke-Davis, Morris Plains, NJ) to prevent bleeding at the injection site. Mice were bled at the indicated times via retroorbital phlebotomy using sterile 100 μL capillary pipettes. Blood was immediately placed into citrate buffer (at 0ºC) to prevent coagulation and plasma was prepared by centrifugation. Plasma was frozen immediately on dry ice then stored at –80ºC for future testing.

Adenovirus production. Av3H8101 is a third-generation vector derived by deleting the regions E1, E2A, and E3 from human serotype 5 adenovirus, and has been described previously (17). Briefly, the human FVIII insert is a B-domain deleted cDNA cloned into the Av3 genome adjacent to the mouse albumin promoter. Av3nBg is a similar vector encoding a nuclear targeted β-galactosidase cDNA, with the exception that expression of this transgene is controlled by the Rous sarcoma virus (RSV) promoter (13). Av3null is similar to the Av3nBg vector construct, but lacks a transgene insert. All vectors used in these studies were propagated *in vitro* in human A549 lung adenocarcinoma packaging cells, stably transfected with E1 and E2a (AE1-2a, Ref. 13), and purified by CsCl density gradient centrifugation as described previously (17).

FVIII activity assay. Biologically active human FVIII was measured using the Coatest chromogenic bioassay as described by the kit manufacturer (Chromogenix AB, West Chester, OH). Typically, three dilutions of plasma were made (10-, 100-, and 1000-fold) for the determination of FVIII activity. A standard curve was generated using purified human FVIII (George King Bio-Medical Inc., Overland Park, KS). Absorbance was read at 405 nm using a Spectramax automated microtiter plate reader (Molecular Devices, Sunnyvale, CA). Data were analyzed using SoftmaxPro software (Molecular Devices). Plasma from the FVIII exon 16 knockout mice was used as a negative control, and normal C57Bl/6 mouse plasma served as a positive control. FVIII knockout mouse plasma contains <1% FVIII activity compared to normal mouse plasma using this Coatest assay (17). The limit of sensitivity of this assay with mouse plasma is approximately 25 mU/mL.

In vitro dose–response assay. The human hepatocarcinoma cell line, HepG2 (catalog No. HB-8065, American Type Culture Collection, Manassas, VA), was cultured in EMEM containing 10% heat-inactivated fetal bovine serum (BioWhitaker, Walkersville, MD), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids. AE1-2a cells were cultured in DMEM containing 10% heat-inactivated fetal bovine serum. Cells were seeded on 96-well culture plates (Costar, Corning, NY) at 1×10^4 cells/well. After overnight incubation to allow attachment to the plates, cells were transduced with the indicated number of particles per cell of Av3H8101 or Av3nBg for 60 min at 37°C then the viral infection media was removed from the wells. Fresh medium was added and the cells were incubated for 24 h in a 37°C, 5% CO_2 incubator. FVIII activity was measured in culture supernatants using the Coatest assay. The limit of sensitivity of the assay using culture supernatant is approximately 10 mU/mL. Av3nBg-transduced cells were washed once with PBS, fixed with 0.5% glutaraldehyde (Sigma Chemical Co., St. Louis, MO, diluted in PBS) for 10 min, then washed with PBS and stained for β-galactosidase expression by incubating with X-gal stain (5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM magnesium chloride, 1 mg/mL X-gal, Sigma Chemical Co.). Cells staining blue were enumerated by light microscopy and the percent transduced cells was calculated.

Assay for liver-specific enzyme activities. To evaluate vector toxicity in mice that received Av3 vectors, blood was collected on day 10 via retroorbital bleeding and citrated plasma was prepared by centrifugation, then frozen at –80ºC. Samples were submitted to Ani-Lytics, Inc. (Gaithersburg, MD) and analyzed for the presence of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALK PHOS). Untreated littermates were used for control enzyme levels.

Lymphoproliferative assay. Hemophiliac mice were treated with adenoviral vectors as described in the legend to Fig. 3. Thirteen days later, spleens were removed and pooled from each group. A single cell suspension was prepared from which T lymphocytes were purified by Ficoll density gradient centrifugation followed by nylon wool separation. The purified T cells were stimulated *in vitro* in a 37°C, 5% CO_2 incubator (1×10^5 T cells mixed with 5×10^5 irradiated spleen cells from a naive hemophiliac mouse as the APC monolayer) for 5 days in 96-well plates with graded doses of either purified recombinant human BDD-FVIII protein (kindly provided by Genetics Institute, Cambridge, MA) or Av3nBg vector at the indicated particles per cell, and T cell proliferation was measured via [^{3}H]thymidine (1 μCi/well, Amersham-Pharmacia Biotech, Piscataway, NJ) incorporation during the final 18 h of incubation. Cells were harvested on glass fiber filter mats using a cell harvester (TomTec, Hamden, CT) and tritium decay was counted using a β-counter (Perkin–Elmer Life Sciences, Gaithersburg, MD).

CTL culture and cytotoxicity assay. Hemophiliac mice were treated with adenoviral vectors as described in the legend to Fig. 3. Thirteen days later, spleens were removed and pooled from each group. A single cell suspension was prepared from which 1×10^8 lymphocytes were placed in T-75 culture flasks containing 20 mL of RPMI complete medium in the presence of either 30 nM purified recombinant human BDD-FVIII (Genetics

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Institute) or 1 × 10^9 vector particles of Av3nBg to approximate an m.o.i. = 10 in a 37°C, 5% CO_2 incubator. After 5 days, viable T cells were recovered by Ficoll density gradient centrifugation, washed and counted to test for cytotoxic activity toward target cells expressing either human FVIII antigens or adenovirus antigens. Briefly, MCA205 target cells (H-2^b haplotype, class I^+ fibrosarcoma cell line derived from B6 mice, kindly provided by Dr. James Mulé, Surgery Branch, National Cancer Institute, Bethesda, MD) were either mock treated, transfected (using Lipofectamine, GibcoBRL, Rockville, MD) with a plasmid encoding human FVIII (5 µg of pAvS6H81 for 6 × 10^6 cells, resulting in typically >500 mU/mL of FVIII activity in supernatants) or, transduced with Av3nBg (m.o.i. = 1000, resulting in typically >80% cell transduction) and incubated for 18 h to allow expression and presentation of FVIII or adenovirus antigenic peptides on MHC class I molecules. Target cells were harvested, labeled for 90 min with 250 µCi of 51-chromium (Amersham-Pharmacia Biotech), then washed and counted. In 96-well U-bottom plates, target cells (1 × 10^4/well) were mixed with graded numbers of effector T cells for 4 h at 37°C, then the supernatants were harvested and gamma emission was measured using a gamma counter (Perkin–Elmer Life Sciences). The percent specific lysis was calculated according to the equation (unknown cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm) × 100.

Anti-FVIII antibody assay. The ELISA, designed to measure human FVIII-specific antibodies, was performed by first incubating purified recombinant human BDD-FVIII (Genetics Institute) overnight on 96-well Immulon-4 plates (Dynatech, Chantilly, VA) at 4°C (100 µL/well at 3 U/mL, in 50 mM carbonate-bicarbonate buffer, pH 9.0). Plates were washed twice with TBS (20 mM Tris–HCl, pH 7.4 containing 140 mM NaCl), then 200 µL of TBS/BSA blocking buffer (TBS containing 0.5% bovine serum albumin) was added for 1 h at room temperature. Plates were rinsed once with TBS, then 100 µL of mouse plasma samples, appropriately diluted in TBS/BSA, were added to wells and incubated overnight at 4°C. Plates were washed three times with TBST (TBS containing 0.1% Tween 20), then the detection antibody, alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (Southern Biotechnology Associates, Inc., Birmingham, AL) diluted 1:3000 in TBS/BSA, was added (100 µL/well) and the plates were incubated at room temperature for 2 h. Plates were washed three times with TBST, then *p*-NPP substrate (Calbiochem, La Jolla, CA) was added (100 µL/well) and the assay was developed for approximately 10–20 min. The enzymatic reaction was stopped by the addition of 20 µL of 1 N NaOH and the absorbance at 405 nm was read using a Spectramax automated microplate reader. The plasma concentration of anti-FVIII antibody was calculated by the SoftmaxPro software program from a standard curve generated by a monoclonal mouse anti-human FVIII antibody, MAb 413 (34). The limit of sensitivity of the ELISA was approximately 20 ng/ml using mouse plasma samples.

Neutralizing anti-adenovirus antibody assay. To evaluate the induction of neutralizing anti-adenovirus antibodies in mice, serum was tested for the ability to inhibit cellular transduction by a control adenovirus *in vitro*. Briefly, AE1-2a cells were seeded at 1 × 10^4 cells/well on a 96-well plate and incubated overnight. The following day, Av3nBg vector was diluted to result in a constant m.o.i. = 10, then pre-incubated with serial dilutions of the mouse serum for 1 h at 37°C. The mixtures (in a total volume of 60 µL) were then added to the cells for 1 h at 37°C to facilitate cellular transduction, the supernatants were aspirated, and the cells were refed with fresh media and incubated at 37°C. After 18 h, cells were washed with PBS, fixed with 0.5% glutaraldehyde for 10 min, then stained for β-galactosidase expression as described above. Cells staining blue were enumerated and the serum dilution at which 50% inhibition of cellular transduction was observed is reported as the anti-adenovirus neutralizing antibody titer. Control wells included untransduced cells, cells transduced at an m.o.i. = 10 in the absence of any serum, and cells transduced at an m.o.i. = 10 in the presence of a known high titer mouse serum containing neutralizing adenovirus antibodies.

Southern blot analysis. Genomic DNA from mouse livers was prepared using QIAmp Tissue Kit (Qiagen, Valencia, CA). A total of 10 µg of each DNA sample was digested with *Bam*HI for FVIII analysis or *Sph*I for adenoviral vector analysis, and subjected to Southern blotting. The probes, prepared by random oligonucleotide priming with [α-^{32}P]dATP, contained FVIII cDNA sequences corresponding to nucleotides 73 to 1345, or adenoviral (serotype 5) sequences corresponding to nucleotides 3661 to 5137. The copy number control standards were prepared by adding 1500, 600, or 60 pg of viral DNA, equivalent to 25, 10, and 1 vector copy per cell, respectively, to 10 µg of control mouse liver genomic DNA digested with *Bam*HI or *Sph*I. Genomic DNA from untreated mouse liver was used as a negative control. The band intensities were quantitated with a Molecular Dynamics PhosphorImager (Sunnyvale, CA).

RESULTS

The development of human adenovirus for use in gene therapy has resulted in several technological advances for establishing and propagating new generations of adenoviral vectors *in vitro*. New vectors are routinely tested in cultured cell lines for expression before they are tested in animal models. This report describes differences we have observed between the *in vitro* and *in vivo* dose responses using an adenovirus vector encoding B-domain deleted human Factor VIII (Av3H8101).

As shown in Fig. 1, the relationship between the Av3H8101 adenovirus particle number used to transduce HepG2 or AE1-2a cells *in vitro* and the FVIII activity detected in the culture supernatant after a 24-h period is linear for three log units of virus particle for AE1-2a cells, and exponential for two log units of vector for HepG2 cells (Fig. 1A). These results were not unique to the Av3H8101 vector constructed with the albumin promoter since a similar Av3 adenovirus vector encoding a nuclear β-galactosidase gene under the control of the RSV promoter (Av3nBg) resulted in a linear dose–response curve in AE1-2a cells and a sigmoidal dose–response curve in HepG2 cells (Fig. 1B). Thus, by two different assays, functional expression of a transgene and cellular transduction efficiency, no *in vitro* threshold dose effect was observed with two adenoviral vectors in either of these two cell lines.

Unlike the *in vitro* dose–response curves, the *in vivo* dose response displayed a threshold dose response at low doses, as demonstrated in Fig. 2A. Systemic delivery of vector doses of 6 × 10^{10}, 4 × 10^{10}, or 2 × 10^{10} particles per mouse resulted in linear FVIII expression levels. By contrast, mice injected with a dose of 1 × 10^{10} Av3H8101 particles resulted in an undetectable FVIII activity level as measured by the Coatest assay 10 days after the vector administration. This lack of activity is typically observed in plasma from the naive FVIII knockout mice. Thus, a twofold difference in vector dose revealed a threshold level for transgene expression in the hemophiliac mice. Also unlike the *in vitro* results, there is a narrow range (less than one log unit) of adenovirus vector dose that results in therapeutic, or even detectable, levels of FVIII activity. This threshold level between 1 and 2 × 10^{10} vector particles per mouse for transgene expression detection has been observed in similar *in vivo* dose response studies using adenovirus encoding human Factor IX (Dr. Ted Smith, personal communication).

We investigated the nature of this threshold effect by

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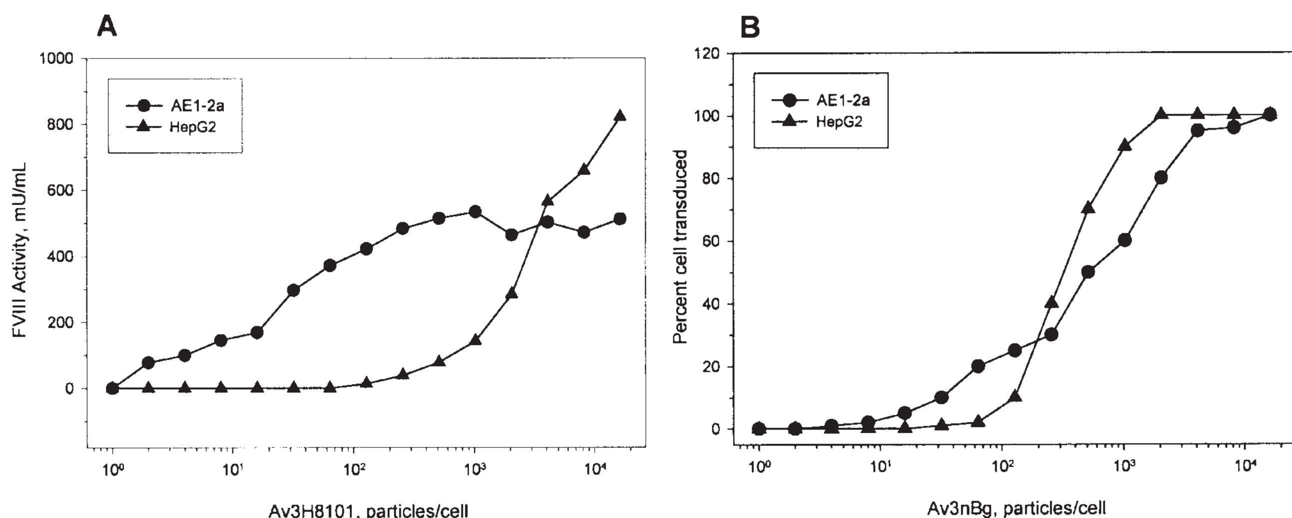

FIG. 1. *In vitro* dose–response curve for Av3 vectors in HepG2 or AE1-2a cells. Cells were infected with Av3H8101 (A) or Av3nBg (B) at the indicated vector particle/cell ratio. After 24 h, cell supernatants were harvested and assayed for FVIII activity by the Coatest assay or, cells were stained for β-galactosidase expression.

injecting into mice admixtures of adenovirus resulting in a total particle load of 6×10^{10} using stepwise mixtures of vector encoding FVIII and an identical adenovirus vector without a transgene insert, the Av3null vector. Figure 2B demonstrates that injecting into mice an admixture containing 1×10^{10} particles of Av3H8101 plus 5×10^{10} particles of Av3null vector resulted in detectable levels of FVIII activity (155 mU/mL) which were not measurable in mice injected with 1×10^{10} particles of the FVIII encoding vector alone (Fig. 2A). Interestingly, administration of admixtures of 2×10^{10} or 4×10^{10} particles of Av3H8101 with Av3null to total 6×10^{10} particles did not appear to alter the FVIII activity levels measured compared to mice that received either 2×10^{10} or 4×10^{10} particles of Av3H8101 vector alone (compare 480 mU/mL to 481 mU/mL for mice that received 2×10^{10} Av3H8101 particles, and 972 to 1100 mU/mL for mice that received 4×10^{10} Av3H8101 particles). A similar vector admixing experiment was repeated with different production lots of both Av3H8101 and Av3null vectors with essentially identical results, verifying the *in vivo* threshold dose phenomenon and precluding any subtle

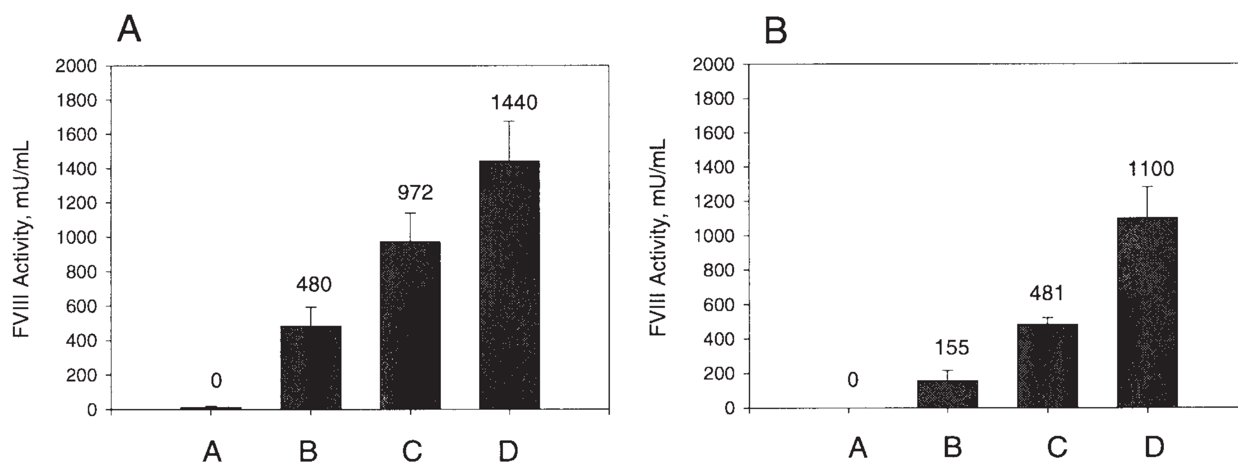

FIG. 2. *In vivo* dose–response curve for Av3 vectors in hemophiliac mice. Mice were injected via tail vein with Av3H8101 vector (A) or, an admixture of Av3H8101 plus Av3null vectors (B). On day 10, mice were bled and plasmas were tested for FVIII activity by the Coatest assay. (A) Group A, 1×10^{10} particles Av3H8101; group B, 2×10^{10} particles Av3H8101; group C, 4×10^{10} particles Av3H8101; group D, 6×10^{10} particles Av3H8101. (B) group A, 6×10^{10} particles Av3null; group B, 1×10^{10} particles Av3H8101 plus 5×10^{10} particles Av3null; group C, 2×10^{10} particles Av3H8101 plus 4×10^{10} particles Av3null; group D, 4×10^{10} particles Av3H8101 plus 2×10^{10} particles Av3null. Data represent means of five mice in each group with the standard error of the mean. Numbers above the error bars indicate mean FVIII activities for each group.

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TABLE 1
Serum Levels of Liver Enzymes from Mice Injected with Av3 Vectors

Treatment group	AST (U/L)	ALT (U/L)	ALK PHOS (U/L)
1×10^{10} Av3H8101	55.4 (14.2)	11.8 (6.6)	115 (24.2)
2×10^{10} Av3H8101	64.6 (17.9)	11.8 (3.1)	94 (26.5)
4×10^{10} Av3H8101	73.4 (11)	10.2 (7.1)	107.4 (22.5)
6×10^{10} Av3H8101	81.2 (25.4)	10 (3.2)	121 (13.1)
6×10^{10} Av3null	62.4 (28)	14 (4.5)	105.4 (29)
1×10^{10} Av3H8101+ 5×10^{10} Av3null	60 (10.8)	9 (6.8)	116 (32.4)
2×10^{10} Av3H8101+ 4×10^{10} Av3null	83 (26.9)	22.7 (5.7)	117.3 (23.4)
4×10^{10} Av3H8101+ 2×10^{10} Av3null	74.4 (31.4)	17 (4.8)	105.4 (25.9)
Untreated littermates	129 (69)	40 (17)	109 (32)
Normal mouse range	72–288	24–140	45–222

Note. Data represent means (± standard deviation) of five mice per group. AST, aspartate transaminase; ALT, alanine transaminase; ALK PHOS, alkaline phosphatase; U/L, units per liter. Untreated littermates were bled on the same day as the treated groups; normal mouse range represents the range for all mouse strains tested by Ani-Lytics, Inc.

differences in vector quantitation methods which might have yielded variable results (data not shown). Thus, by administration of a higher total particle number into these mice, the threshold effect at the low FVIII vector dose (1×10^{10} particles) was overcome.

To gain insight into the nature of the *in vivo* threshold effect observed using adenoviral vectors, we examined whether any liver toxicity had occurred in these mice. Evidence of liver toxicity in animals injected with various doses of adenovirus might reveal a possible mechanism for the threshold level of transgene expression. Table 1 lists the serum levels of three major liver enzyme activities normally used as a readout for hepatotoxicity. The data showed no elevation of liver enzyme levels on day 10 associated with any of the doses injected compared to values from normal untreated littermates. Thus, the possibility of adenovirus-induced hepatotoxicity causing a dose-dependent expression threshold effect can be excluded at the doses reported here.

The contribution to the threshold effect by immune responses induced by adenovirus-mediated gene therapy was evaluated. Our hypothesis was that adenovirus-specific immune responses might create a unique cytokine milieu in the liver, or recruit specific effector cells that would affect transgene expression. To this end, T lymphocytes were recovered from mouse spleens 2 weeks after vector administration and tested *in vitro* for antigen-specific functional activities. Figure 3 demonstrates a weak proliferative response to adenovirus-derived antigens (Fig. 3A), although there does not appear to be a correlation between the vector dose and the magnitude of T cell proliferation. In separate experiments, proliferation was demonstrated to be $CD4^+$ T cell-mediated by the inclusion of anti-CD4 antibodies during the assay (data not shown). In contrast, no T cell proliferation was detected in response to FVIII-derived antigens (Fig. 3B), a result that has been reproduced in other experiments in our laboratory which suggests that endogenously expressed human FVIII may not be immunogenic in these hemophiliac mice (Ref. 17 and J. A. Bristol and S. Connelly, unpublished results).

Spleen-derived T cells from mice injected with the highest vector dose were also restimulated *in vitro* for 5 days with either adenovirus or purified recombinant

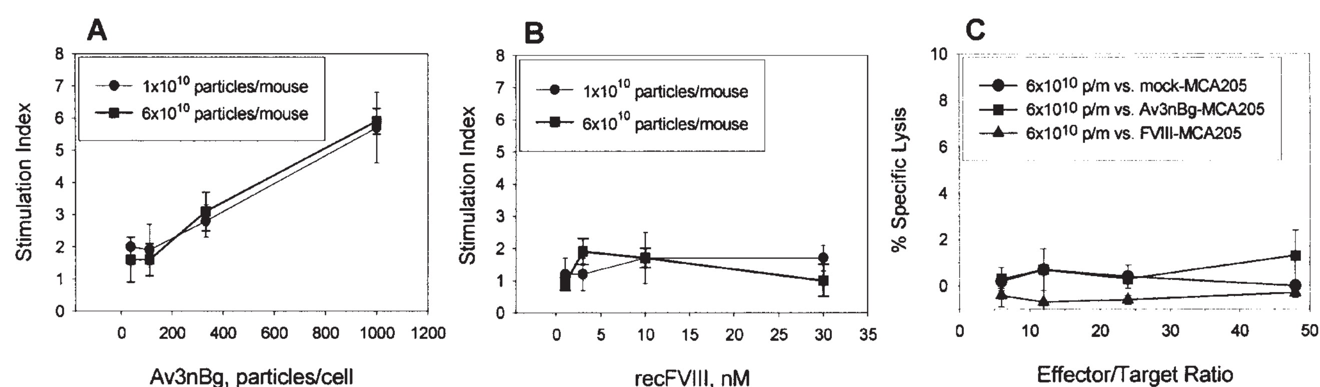

FIG. 3. Cellular immune responses in mice injected with Av3H8101 vector. Hemophiliac mice (five per group) were injected via tail vein with Av3H8101 vector with the doses indicated in the graph inset. On day 13, mice were sacrificed and their splenocytes prepared and tested. (A) Lymphoproliferation against Av3nBg; (B) lymphoproliferation against recombinant human BDD-FVIII (recFVIII). The data are presented as the stimulation index over T cells from each group incubated in the absence of antigen and represent the mean of triplicate wells with the standard error of the mean. (C) Cytotoxic activity from mice injected with 6×10^{10} particles of Av3H8101 vector. Splenocytes were stimulated *in vitro* with either adenovirus (Av3nBg) or recombinant human BDD-FVIII. After 5 days, viable cells were recovered and tested in a standard 4-h chromium release assay for cytotoxicity against MCA205 target cells that were either mock-treated (circles), pretransduced with Av3nBg (squares), or pretransfected with a mammalian cell expression plasmid encoding human FVIII (triangles), as indicated in the graph inset. Data are presented as the percent specific lysis with the standard error of the mean of triplicate wells.

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human BDD-FVIII and tested for cytotoxic activity. No cytotoxicity was measured against MCA205 target cells presenting either adenoviral- or FVIII-derived antigens from mice injected with any dose of the Av3H8101 vector (Fig. 3C). In both the lymphoproliferation assay and the cytotoxicity assay, control experiments (conconavalin A stimulation or redirected cell lysis) confirmed functionally that T cells derived from these mice were otherwise competent compared to untreated mice, ruling out the possibility that T cells from adenovirus-treated mice were functionally compromised. Furthermore, the MCA205 target cells used were shown to be permissive to Av3nBg infection, and to express FVIII activity upon transfection with a FVIII mammalian cell expression plasmid, demonstrating the ability of the MCA205 cell line to transiently express exogenous genes for MHC class I presentation. The results show that the hemophiliac mice injected systemically with these doses of Av3H8101 do not activate a detectable cellular response to the FVIII expressed endogenously, and induce only a weak dose-independent $CD4^+$ T cell proliferative response to the adenovirus vector.

In addition to cellular immune responses, evidence for a humoral immune response was evaluated in the serum from mice injected with the graded doses of adenovirus. Figure 4 shows that 10 days after one injection of the indicated particles of Av3H8101, no humoral immunity is detected which reacts with endogenously expressed human FVIII compared to serum from naive mice used as a negative control (Fig. 4A). A positive control serum from mice immunized with recombinant human FVIII mixed with adjuvant is included to show that these mice are immunocompetent to mount an antibody response to human FVIII under certain conditions. By contrast, ten days after vector administration there is evidence for the induction of neutralizing antibodies to adenoviral antigens, which appears not to be dose-dependent (Fig. 4B). Sera from mice immunized with Av3nBg mixed in adjuvant, and from naive untreated mice, were used as the positive and negative control sera, respectively. Thus, in the absence of antigen-specific immune responses to FVIII expressed by these doses of adenovirus vector, there does not appear to be a correlation between induced immunity and the FVIII expression threshold effect.

An alternative explanation for the observed threshold effect predicts that some critical number of adenovirus transduction events per cell or, a total percentage of transduced liver cells, may be necessary before transgene expression can be detected. To test this directly in an independent experiment, mice were injected with the threshold level dose of Av3H8101 (1.2×10^{10} particles in this experiment) either alone or, admixed with 4.8×10^{10} particles of Av3null vector resulting in a total dose of 6×10^{10} vector particles per mouse. Southern analysis of DNA from the livers of these mice is shown in Fig. 5. Figure 5A shows strong signals representing human FVIII DNA from mice injected with 6×10^{10} particles of Av3H8101 and barely detectable signals from mice injected with 1.2×10^{10} particles of Av3H8101, data

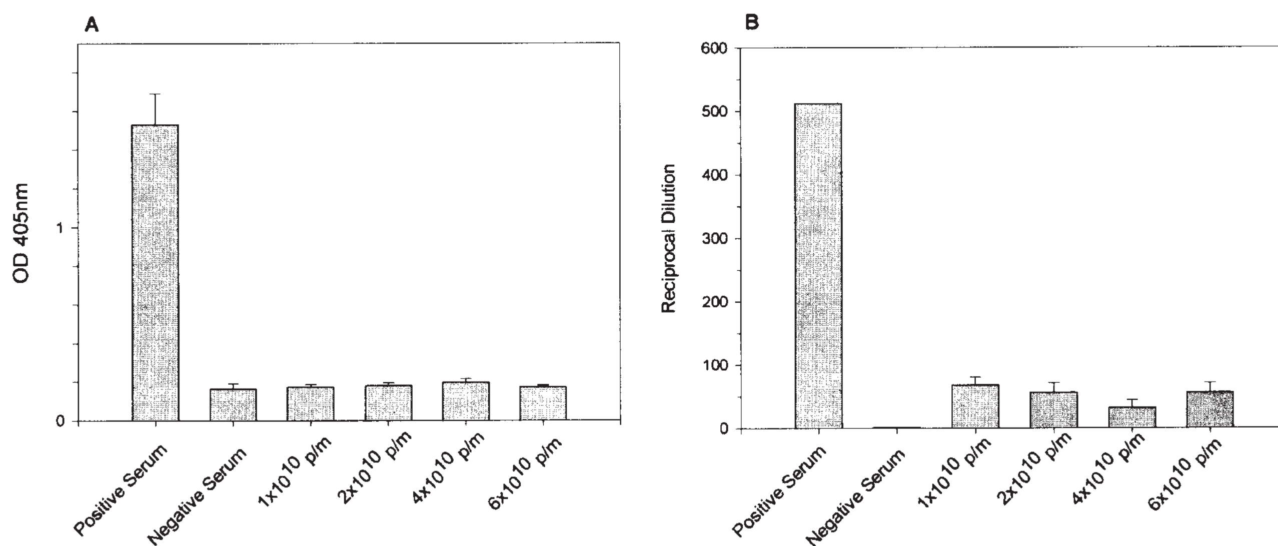

FIG. 4. Humoral responses to human FVIII and adenovirus antigens in mice injected with Av3H8101. Ten days after injection with the indicated doses of Av3H8101, mice were bled and sera were tested for antibodies. (A) ELISA to detect anti-human FVIII-specific antibodies. Data represent mean OD (405 nm) values from five mice per group at the least dilution tested (1:4). Positive control serum was derived from hemophiliac mice immunized with BDD-FVIII in adjuvant whereas negative control serum was from naive hemophiliac mice. (B) The presence of neutralizing antibodies in mouse serum inhibits cellular transduction by a control adenovirus (Av3nBg). Data are presented as the serum dilution that results in >50% inhibition of cell transduction by Av3nBg vector and are the mean dilutions for each group with the standard error of the mean. Positive control serum was derived from mice immunized with Av3nBg in adjuvant whereas negative control serum was from naive hemophiliac mice. In each panel, treatment groups are identified along the abscissa.

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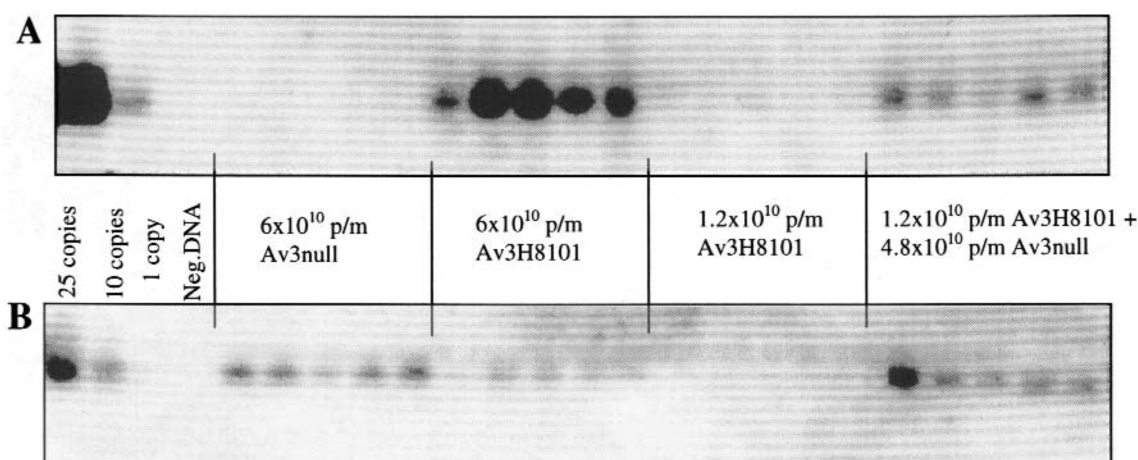

FIG. 5. Southern analyses of livers from mice injected with Av3 vectors. Ten days after systemic injection of the indicated doses of either Av3H8101, Av3null, or an admixture of both vectors, genomic DNA from mouse livers was prepared and subjected to Southern analysis using probes derived from the FVIII cDNA (A) or the Av3 vector backbone DNA (B). Lanes containing standards (25, 10, or 1 viral genome copy equivalent) are indicated. Liver DNA prepared from an untreated hemophiliac mouse served as a negative control. DNA from vector treated mice (five per group) were electrophoresed in separate lanes and are labeled in groups as depicted between the panels.

which correlate well with the FVIII activity assay (Fig. 2A). However, mice injected with 6×10^{10} total particles of the admixture of Av3H8101 plus Av3null resulted in moderate FVIII DNA signals in all mice (Fig. 5A). Image analysis of the Southern blot indicated that mice which received 6×10^{10} Av3H8101 vector particles contained approximately 3.5-fold higher levels of FVIII DNA in their livers compared to mice that received the admixture of adenovirus vectors with only 1.2×10^{10} particles of Av3H8101. Thus, the addition of the Av3null vector to give a higher total vector dose contributed to the increased cellular transduction by the Av3H8101 vector, allowing for FVIII expression in the livers of the hemophiliac mice. The vector DNA control Southern blot shown in Fig. 5B shows that mice injected with a total dose of 6×10^{10} Av3 vector particles transduced similar numbers of liver cells (less than a 2-fold quantitative difference between the three groups), but mice injected with 1.2×10^{10} Av3 vector particles failed to transduce detectable amounts of liver cells. Overall, the Southern blot analysis of mice injected with the admixture of vectors to result in a higher total adenovirus load supports the FVIII activity data, suggesting that a critical minimum adenoviral vector dose may be necessary to achieve liver transduction and therefore therapeutic transgene expression levels.

DISCUSSION

We describe in this report an *in vivo* threshold dose-response phenomenon, which was not observed *in vitro*. The *in vitro* dose response curves were linear, exponential, or sigmoidal over a wide range (2–3 log units) with two different adenoviral vectors and two different cell lines shown in this report (Fig. 1). Although the curves from the *in vitro* data are somewhat different, in no case was there a threshold dose effect such that small incremental changes in vector dose (twofold increases) resulted in a large effect on transgene expression. These differences are probably not due to the vectors themselves but to other factors specific to the cell lines such as differences in cell surface expression of the adenovirus receptor, host cell limitations for transgene expression due to the promoter used in the vector, or the possibility that some vector replication and cell lysis might occur within 24 h in the AE1-2a cells that express the E1 and E2a proteins. Nonetheless, the *in vivo* dose response result with the third-generation Av3H8101 vector (E1, E2a, E3 deleted) was very different, with a threshold level for transgene expression observed between doses of approximately $1–2 \times 10^{10}$ particles per mouse ($5–10 \times 10^{11}$ particles/kg). The FVIII activity observed from mice injected with 1×10^{10} particles or 2×10^{10} particles of Av3H8101 escalates from an undetectable level to nearly 500 mU/mL. Thus, a small (twofold) increase in vector dose resulted in a large disproportionate effect in transgene expression. This threshold effect cannot be attributed to the sensitivity of the Coatest assay used to measure FVIII activity since the assay can detect FVIII levels as low as 25 mU/mL in plasma and as low as 10 mU/mL in culture supernatants (Fig. 1). At doses of 2×10^{10} particles or higher, but within a threefold range, the FVIII activity detected in the plasma of hemophiliac mice was dose-dependent in a linear fashion (Fig. 2). It should be noted that a twofold higher dose of FVIII vector (1.3×10^{11} particles/mouse) resulted in FVIII expression that was linear in comparison to the highest dose of 6×10^{10} particles/mouse reported here (43). Another possible explanation for differences in FVIII detection at the threshold dose is the FVIII clearance rate from mouse plasma. If

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FVIII is removed or degraded such that we could not detect activity in mice that received 1×10^{10} vector particles, but we could detect FVIII activity in mice that received 2×10^{10} vector particles, then this might contribute to an explanation for the threshold effect. However, this possibility can likely be excluded since the Southern blots showed no, or barely detectable, signals from mice that received 1.2×10^{10} vector particles (Fig. 5).

A similar vector dose threshold effect was observed in the hemophiliac mice with a third-generation adenoviral vector encoding canine FVIII (44). Furthermore, in a nonhuman primate model in which similar doses of a FVIII adenoviral vector were administered (3×10^{12} particles/kg; equivalent to 6×10^{10} particles/mouse), physiological plasma levels of FVIII were observed, while only a fivefold lower dose yielded neither FVIII-specific RNA nor FVIII protein (45). Together, these data suggest that the adenoviral *in vivo* dose threshold effect may be a general phenomenon that has important implications in defining a therapeutic window in clinical applications.

The engineering of new adenoviral vectors has generally yielded less toxic viruses. The Av3 adenovirus used in this study is a serotype 5, E1-, E2a-, E3-deleted vector expressing human FVIII under the control of the albumin promoter. The deletion of early regulatory regions of adenovirus is believed to reduce both its direct toxicity and its ability to induce immune responses (11, 14, 23). In support of this notion, we did not observe any remarkable liver toxicity associated with the Av3 adenovirus at the doses reported here in hemophiliac mice (Table 1).

The data presented here demonstrate that although there is evidence for weak humoral and $CD4^+$ T cell induction specific for adenoviral antigens in the mice after a single systemic injection, the responses were not dose-dependent. This may not be surprising since the range of doses administered to mice was narrow (sixfold range), and adenovirus was delivered in a single systemic injection without adjuvant. Several reports have detailed the cytotoxic T lymphocyte (CTL) immune response to adenoviral vector antigens in different experimental models (24–26). In contrast, we did not detect adenovirus-specific $CD8^+$ CTL responses in the hemophiliac mice after a single administration of the Av3 vector. The reason for this difference is unclear but may be attributed to several parameters including the version of the adenoviral vector injected, different mouse strains with diverse immune responses to adenovirus (35), and variable *in vitro* stimulation and assay conditions used to test for cytotoxicity. Nonetheless, we observed both $CD4^+$ T cell (Fig. 3) and antibody (Fig. 4) responses to adenovirus-specific antigens, demonstrating that these mice are immunocompetent and this version of adenoviral vector is immunogenic after a single injection.

Immunity to the transgene (whether foreign or not) is not always observed during *in vivo* applications of adenovirus-mediated gene therapy. Unlike some reports on the immunogenicity of human FVIII in hemophiliac mice (36, 37), we observed neither antibodies nor cellular immune responses to human FVIII delivered by a single injection of adenoviral-mediated gene therapy (Figs. 3 and 4). The use of third-generation Av3 vectors, combined with the liver-specific albumin promoter to express FVIII, which normally is expressed in the liver, probably contributes to the lack of detectable FVIII-specific immune responses in these mice. The application of tissue-specific promoters in adenovirus-mediated gene therapy has been reported elsewhere (19, 20) and supports the hypothesis that tissue-specific promoters lead to decreased transgene immunogenicity and prolonged transgene expression. Possible mechanisms may be that albumin promoter-driven expression of transgenes may interfere least with normal liver cell functions and thereby cause the least disruption to the cell (compared to a viral promoter, for example), or that extrachromosomal FVIII expression in the liver may be less immunogenic than FVIII expression in other tissues that do not normally express this coagulation factor. Alternatively, the specific liver cell type transduced by adenovirus may not represent an appropriate antigen presenting cell for immune response activation. This possibility could be tested by examination of adenovirus-transduced liver cells for MHC molecule expression as well as expression of a number of costimulation molecules known to activate immune responses.

Injections of the admixture of Av3H8101 and Av3null vectors revealed that a higher total dose of adenovirus was necessary to achieve detectable levels of liver transduction, which correlated with the detection of transgene expression. In the absence of dose-dependent hepatotoxicity or potent specific immunity to the vector, an alternative explanation for the dose threshold effect could be an acute inflammatory response depleting some percentage of the adenovirus nonspecifically within hours after vector injection. It has been demonstrated that upon administration of adenovirus, the first few hours are met by rapid increases in serum levels of inflammatory cytokines such as interleukin-6, interleukin-8, and interferon-γ, as well as several inflammatory chemokines. These inflammatory molecules in turn recruit and activate cellular defense mechanisms including macrophage, basophils, and mast cells which can induce IgE-dependent acute phase anaphylaxis (23, 38). The hypothesis that nonspecific inflammation mechanisms clear adenovirus upon administration is supported by reports which examined acute phase responses to adenovirus administered either systemically (39, 46) or into the lungs (40, 41). It was estimated that approximately 90% of an adenoviral vector administered intravenously to BALB/c mice was cleared from the circulation within the first 5 min, mediated primarily in the liver by Kupffer cells, a type of tissue macrophage (46). Furthermore, immunodeficient and immunocompetent mice were shown to clear virus similarly (41) and have similar inflammatory cytokine and chemokine profiles

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in response to intratracheal adenovirus administration (40), suggesting that although immunodeficient mice cannot launch a specific immune response against adenoviral antigens, they are capable of inducing a nonspecific acute inflammatory response toward the vector. One hypothesis that follows from these observations is that acute non-specific inflammatory responses eliminate the majority of circulating vector within hours after systemic administration before the adenovirus can transduce hepatocytes. This inflammatory response, however, can be overcome by large adenovirus doses, potentially by saturating the Kupffer cells (46) and allowing the excess vector to transduce hepatocytes. In these mice, it would appear that a dose of 1×10^{10} particles of Av3H8101 represents a threshold level, or saturation level for the inflammatory response. However, doses above approximately 5×10^{11} particles per mouse are hepatotoxic (43), leaving a narrow window of vector dose to deliver therapeutic levels of transgene product. This hypothesis is supported by a recent report that used an E1-deleted adenovirus vector encoding human Factor IX in rhesus macaques to demonstrate a dose-dependent acute phase inflammatory response to vector administration followed by transient Factor IX expression whose decrease correlated inversely with Factor IX antibody development (8). These authors also suggest from their studies that there is probably a narrow therapeutic window for adenovirus-mediated gene transfer. Thus, in the context of future phase I clinical trials involving adenovirus for gene therapy, the appropriate vector dose should be a critically important consideration.

Although the threshold level of transgene expression was overcome by administering a higher total dose of vector (1×10^{10} particles of Av3H8101 plus 5×10^{10} particles of Av3null), it was surprising that the admixture of 2×10^{10} particles of Av3H8101 plus 4×10^{10} particles of Av3null did not similarly increase FVIII expression compared to mice injected with 2×10^{10} particles of Av3H8101 alone. One possible explanation may be that although the total admixed dose was constant for both groups of mice (6×10^{10} particles), the ratios of the two vectors were different (1:5 versus 2:4) and the animal model may not be sensitive enough to distinguish this difference.

In summary, we report here an *in vivo* dose threshold effect in adenovirus-mediated gene therapy. While there are no published reports describing this particular phenomenon, there is anecdotal evidence which supports our observations. For example, adenoviruses encoding human coagulation Factor IX, administered to C57Bl/6 mice displayed a similar dose threshold effect (personal communication, Ted Smith, Genetic Therapy, Inc., February 2000). The observations reported here highlight the narrow therapeutic window of adenoviral vector dose for *in vivo* gene therapy, one of many of the considerations necessary for gene therapy to advance from the laboratory to the clinic.

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